

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

*December 06, 2004*

**THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.**

**APPLICATION NUMBER: 10/635,396**

**FILING DATE: August 06, 2003**

**RELATED PCT APPLICATION NUMBER: PCT/US04/25395**

Certified by

Jon W Dudas

Acting Under Secretary of Commerce  
for Intellectual Property  
and Acting Director of the U.S.  
Patent and Trademark Office



**BEST AVAILABLE COPY**

UC Davis Case No. 2003-196-1

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**APPLICATION FOR PATENT**

**ERYTHROCYTIC CELLS AND METHOD FOR LOADING SOLUTES**

Inventors: John H. Crowe  
Fern Tablin  
Nelly M. Tsvetkova  
Zsolt Torok  
Gyana Satpathy  
Denis Dwyre

**Related Patent Applications**

This patent application is related to co-pending patent application Serial No. 10/052,162, filed January 16, 2002. Patent application Serial No. 10/052,162 is a continuation-in-part patent application of co-pending patent application Serial No. 09/927,760, filed August 9, 2001. Patent application Serial No. 09/927,760 is a continuation-in-part patent application of co-pending patent application Serial No. 09/828,627, filed April 5, 2001. Patent application Serial No. 09/828,627 is a continuation patent application of patent application Serial No. 09/501,773, filed February 10, 2000. All of the foregoing patent

applications are fully incorporated herein by reference thereto as if repeated verbatim immediately hereinafter.

### Field of the Invention

Embodiments of the present invention generally broadly relate to living mammalian cells. More specifically, embodiments of the present invention generally provide for the preservation and survival of cells, especially human cells, such as erythrocytic cells.

Embodiments of the present invention also generally broadly relate to the therapeutic uses of cells; and more particularly to manipulations or modifications of erythrocytic cells, such as loading erythrocytic cells with solutes and in preparing dried compositions that can be re-hydrated at the time of application. When cells for various embodiments of the present invention are re-hydrated, they are immediately restored to viability.

The compositions and methods for embodiments of the present invention are useful in many applications, such as in medicine, pharmaceuticals, biotechnology, and agriculture, and including transfusion therapy, as hemostasis aids and for drug delivery.

### Statement Regarding Federal Sponsored Research and Development

Embodiments of this invention were made with Government support under Grant No. N66001-00-C-8048, awarded by the Department of Defense Advanced Research Projects Agency (DARPA). Further embodiments of this invention were made with Government support under Grant Nos. HL57810 and HL61204, awarded by the National Institutes of Health. The Government has certain rights to embodiments of this invention.

### Background of the Invention

A cell is broadly regarded in the art as a small, typically microscopic, mass of protoplasm bounded externally by a semi-permeable membrane, usually including one or more nuclei and various other organelles with their products. A cell is capable either alone or interacting with other cells of performing all the fundamental function(s) of life, and forming the smallest structural unit of living matter capable of functioning independently.

Cells may be transported and transplanted; however, this requires cryopreservation which includes freezing and subsequent reconstitution (e.g., thawing, re-hydration, etc.) after transportation. Unfortunately, a very low percentage of cells retain their functionality after undergoing freezing and thawing. While some cryoprotectants, such as dimethyl sulfoxide, tend to lessen the damage to cells, they still do not prevent some loss of cell functionality.

Trehalose has been found to be suitable in the cryopreservation of cells and platelets. Trehalose is a disaccharide found at high concentrations in a wide variety of organisms that are capable of surviving almost complete dehydration. Trehalose has been shown to stabilize membranes, proteins, and certain cells during freezing and drying in vitro.

U.S. Patent No. 5,827,741, Beattie et al., issued October 27, 1998, discloses cryoprotectants for human cells and platelets, such as dimethylsulfoxide and trehalose. The cells or platelets may be suspended, for example, in a solution containing a cryoprotectant at a temperature of about 22°C and then cooled to below 15°C. This incorporates some cryoprotectant into the cells or platelets, but not enough to

prevent hemolysis of a large percentage of the cells or platelets.

Accordingly, a need exists for the effective and efficient preservation of cells. More specifically, and accordingly further, a need also exists for the effective and efficient cryopreservation of cells (e.g., erythrocytic cells, eukaryotic cells, or any other cells, and the like), such that the preserved cells respectively maintain their biological properties and may readily become viable after storage.

### Summary of Embodiments of the Invention

In one aspect of the present invention, a dehydrated composition is provided having a generally dehydrated composition comprising dried cells selected from a mammalian species (e.g., a human) and being effectively loaded internally (e.g., producing hyper-osmotic pressure on the cells to take up external trehalose via fluid phase endocytosis) with at least about 10 mM of a carbohydrate (e.g., an oligosaccharide, such as trehalose) therein to preserve biological properties during drying and re-hydration. The amount of the carbohydrate inside the dried cells is preferably the amount obtained from maintaining a positive loading gradient or loading efficiency gradient on the cell. When the carbohydrate is trehalose, the amount of trehalose loaded inside the dried cells is preferably from about 10 mM to about 50 mM.

In another aspect of the present invention, a method is provided for loading (e.g., by fluid phase endocytosis) a solute into a cell (e.g., an erythrocytic cell). Embodiments of the invention include disposing a cell in a solution having a solute concentration of sufficient magnitude to produce hyper-osmotic pressure on the cell for transferring a solute (e.g., an oligosaccharide, such as trehalose) from the solution into the cell. The method may additionally comprise preventing a decrease in a loading efficiency gradient in the loading of the solute into the cell. In an embodiment of the invention where the solute comprises an oligosaccharide, the preventing a decrease in a loading efficiency gradient in the loading of the oligosaccharide into the cell may comprise maintaining a concentration of the oligosaccharide in the oligosaccharide solution below a certain concentration, such as below from about 35 mM to about 65 mM, more particularly below a concentration

ranging from about 40 mM to about 60 mM, more particularly further below a concentration ranging from about 45 mM to about 55 mM (e.g., below about 50 mM). In another embodiment of the invention, the preventing a decrease in a loading efficiency gradient in the loading of the oligosaccharide into the cell comprises maintaining a positive gradient of loading efficiency to concentration of the oligosaccharide in the oligosaccharide solution.

The solute concentration includes an extracellular solute concentration for elevating extracellular osmolarity within the solution to a value which is greater than a value of the intracellular osmolarity of the cell. The transferring of the solute is preferably by fluid phase endocytosis and preferably without degradation of the solute. In embodiments of the invention where the cell is an erythrocytic cell and the solute comprises trehalose, a gradient of trehalose (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the solution may range from about 0.130 to about 0.200, particularly for a temperature ranging from about 30° C to about 40° C (e.g., about 37° C). In a further embodiment of the invention, a gradient of trehalose (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the solution ranges from about 0.04 to about 0.12, particularly for a temperature ranging from about 0° C to about 10° C. In yet a further embodiment, a gradient of trehalose (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the solution may range from about 0.04 to about 0.08, or from about 0.08 to about 0.12, particularly for a temperature ranging from about 0° C to about 10° C. The solute solution may have a trehalose concentration ranging from about 320 mM to about 4000 mM, such as including from about 320 mM to about 2000 mM or from about 500 mM to about 1000 mM.

A further embodiment of the invention provides retaining the solute in the cell; more specifically, washing the cell and retaining the solute in the cell during the washing. The washing is with a washing buffer, and retention of the solute in the cell increases from about 25% to about 175% when a buffer concentration (e.g., the osmolarity of all osmotically active particles within the washing buffer solution) increases from about 50% to about 400%, more preferably from about 50% to about 150% when a buffer concentration increases from about 100% to about 300%, and most preferably from about 75% to about 125% (e.g., about 100%) when a buffer concentration increases from about 150% to about 250% (e.g., about 200%). The washing of the cell with a washing buffer includes employing a ratio of an extracellular buffer concentration (mOsm) to an intracellular solute concentration (mM) ranging from about 14.0 to about 4.0, such as from about 12.0 to about 5.0, including from about 9.0 to about 6.0 and from about 8.0 to about 7.0 (e.g., about 7.5).

Additional embodiments of the present invention provide a method for loading trehalose into an erythrocytic cell. The method may comprise disposing an erythrocytic cell in a trehalose solution having a trehalose concentration of at least about 25 % (preferably at least about 50%) greater than the intracellular osmolarity of the erythrocytic cell for loading (e.g., by fluid phase endocytosis) the trehalose into the erythrocytic cell.

The loading of the trehalose from the trehalose solution into the erythrocytic cell may be without degradation of the trehalose, and produces a loaded erythrocytic cell having a gradient of loaded trehalose (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the trehalose solution ranging from about 0.130 to about 0.200. In another embodiment, the loading of the trehalose produces a



loaded erythrocytic cell having a gradient of loaded trehalose (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the trehalose solution ranging from about 0.04 to about 0.12. In a further embodiment, the loading of the trehalose produces a loaded erythrocytic cell having a gradient of loaded trehalose (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the trehalose solution ranging from about 0.04 to about 0.08, or from about 0.08 to about 0.12, depending on the extracellular trehalose concentration and the temperature of the trehalose solution. The trehalose solution may have a trehalose concentration ranging from about 25 % to at least about 1000 % greater than the intracellular osmolarity of the erythrocytic cell, or at least about 50% greater than the intracellular osmolarity of the erythrocytic cell.

A further embodiment of the invention provides retaining the trehalose in the erythrocytic cell; more specifically washing the erythrocytic cell and retaining the trehalose in the erythrocytic cell during the washing.

The washing of the erythrocytic cell is preferably with a washing buffer, and retention of the trehalose in the erythrocytic cell increases from about 25% to about 175% when a buffer concentration increases from about 50% to about 400%, more preferably from about 50% to about 150% when a buffer concentration increases from about 100% to about 300%, and most preferably from about 75% to about 125% (e.g., about 100%) when a buffer concentration increases from about 150% to about 250% (e.g., about 200%). The washing of the erythrocytic cell with a washing buffer includes employing a ratio of an extracellular buffer concentration (mOsm) to an intracellular trehalose concentration (mM) ranging from about 14.0 to about 4.0, more particularly from about 12.0 to about 5.0, including from about

9.0 to about 6.0 and from about 8.0 to about 7.0 (e.g., about 7.5).

Additional embodiments of the present invention provide a method for loading (e.g., by fluid phase endocytosis) an oligosaccharide into cells (e.g., erythrocytic cells) comprising disposing cells in an oligosaccharide solution having an oligosaccharide concentration of at least about 25 % greater than the intracellular osmolarity of the cells for loading oligosaccharide into the cells, and preventing a decrease in a loading gradient in the loading of the oligosaccharide into the cells. In one embodiment of the invention, the preventing a decrease in a loading gradient in the loading of the oligosaccharide into the cells comprises maintaining a concentration of the oligosaccharide in the oligosaccharide solution below a certain concentration, such as below a concentration ranging from about 35 mM to about 65 mM, more particularly below a concentration ranging from about 40 mM to about 60 mM, more particularly further below a concentration ranging from about 45 mM to about 55 mM (e.g., below about 50 mM). In another embodiment the preventing a decrease in a loading gradient in the loading of the oligosaccharide into the cells comprises maintaining a positive gradient of concentration of oligosaccharide loaded into the cells to concentration of the oligosaccharide in the oligosaccharide solution.

These provisions, together with the various ancillary provisions and features which will become apparent to those skilled in the art as the following description proceeds, are attained by the processes and cells of the present invention, preferred embodiments thereof being shown with reference to the accompanying drawings, by way of example only, wherein:

### Brief Description of the Drawings

In the drawings:

Figure 1 graphically illustrates the loading efficiency of trehalose plotted versus incubation temperature of human platelets;

Figure 2 graphically illustrates the loading efficiency (cytosolic concentration divided by the extracellular concentration, the sum multiplied by 100) following incubation as a function of incubation time;

Figure 3 graphically illustrates the internal trehalose concentration of human platelets versus external trehalose concentration as a function of temperature at a constant incubation or loading time;

Figure 4 graphically illustrates the loading efficiency of trehalose into human platelets as a function of external trehalose concentration;

Figure 5 graphically illustrates intracellular trehalose concentration in erythrocytic cells as a function of extracellular trehalose concentration at respective temperatures of 4° C and 37° C;

Figure 6 graphically illustrates the fragility index of erythrocytic cells incubated overnight at respective temperatures of 4° C and 37° C in the presence of and as a function of increasing intracellular trehalose concentrations;

Figure 7 graphically illustrates trehalose uptake (i.e., intracellular trehalose mM) and hemolysis (i.e., % hemolysis) as a function of incubation temperature (°C); and

Figure 8 graphically illustrates intracellular trehalose (mM) as a function of the osmolarity of the washing buffer.

### Detailed Description of Preferred Embodiments of the Invention

Compositions and embodiments of the invention include methods for loading solutes into cells, as well as cells that have been manipulated (e.g., by freeze-drying) or modified (e.g., loaded with a chemical or drug) in accordance with methods of the present invention. The cells may be any type of cell including, not by way of limitation, erythrocytic cells, eukaryotic cells or any other cell, whether nucleated or non-nucleated.

The term "erythrocytic cell" is used to mean any red blood cell. Mammalian, particularly human, erythrocytes are preferred. Suitable mammalian species for providing erythrocytic cells include by way of example only, not only human, but also equine, canine, feline, or endangered species.

The term "eukaryotic cell" is used to mean any nucleated cell, i.e., a cell that possesses a nucleus surrounded by a nuclear membrane, as well as any cell that is derived by terminal differentiation from a nucleated cell, even though the derived cell is not nucleated. Examples of the latter are terminally differentiated human red blood cells. Mammalian, and particularly human, eukaryotes are preferred. Suitable mammalian species include by way of example only, not only human, but also equine, canine, feline, or endangered species.

Broadly, the preparation of solute-loaded cells in accordance with embodiments of the invention comprises the steps of loading one or more cells with a solute by placing one or more cells in a solution having a solute concentration of sufficient magnitude to produce hyperosmotic pressure on the cell for transferring the solute from the solution into the cell. For increasing the transfer or uptake of the solute from

the solute solution, the solute solution temperature or incubation temperature has a temperature above about 25°C, more preferably above 30°C, such as from about 30°C to about 40°C. In another embodiment of the invention, a solute solution (e.g., trehalose solution) has a solute (e.g., trehalose) concentration of at least about 25 %, preferably at least about 50 %, greater than the intracellular osmolarity of the cells for loading the solute into the cells. For various embodiments of the invention, a solute solution has a solute concentration ranging from about 25 % to at least about 1000 % greater than the intracellular osmolarity of the cell. For additional various embodiments of the invention, the solute solution has a solute concentration ranging from about 320 mM to about 4000 mM, preferably from about 320 mM to about 2000 mM, more preferably from about 500 mM to about 1000 mM. The method may additionally comprise preventing a decrease in a loading gradient and/or a loading efficiency gradient in the loading of the solute into the cells. Preventing a decrease in a loading efficiency gradient in the loading of the solute into the cells comprises maintaining a positive gradient of loading efficiency (e.g., in %) to concentration (e.g., in mM) of the solute in the solute solution. Preventing a decrease in a loading gradient in the loading of the oligosaccharide into the cells comprises maintaining a concentration of the solute in the solute solution below a certain concentration (e.g., below a concentration ranging from about 35 mM to about 65 mM, more particularly below from about 40 mM to about 60 mM, or below from about 45 mM to about 55 mM, such as below about 50 mM); and/or maintaining a positive gradient of concentration of solute loaded into the cells to concentration of the solute in the solute solution.

The solute solution may be any suitable physiologically acceptable solution in an amount and under conditions effective to cause uptake or "introduction" of the solute from the solute solution into the cells. A physiologically acceptable solution is a suitable solute-loading buffer, such as any of the buffers stated in the previously mentioned related patent applications, all having been incorporated herein by reference thereto.

The solute is preferably a carbohydrate (e.g., an oligosaccharide) selected from the following groups of carbohydrates: a monosaccharide (e.g., bioses, trioses, tetroses, pentoses, hexoses, heptoses, etc), a disaccharide (e.g., lactose, maltose, sucrose, melibiose, trehalose, etc), a trisaccharide (e.g., raffinose, melezitose, etc), or tetrasaccharides (e.g., lupeose, stachyose, etc), and a polysaccharide (e.g., dextrans, starch groups, cellulose groups, etc). More preferably, the solute is a disaccharide, with trehalose being the preferred, particularly since it has been discovered that trehalose does not degrade or reduce in complexity upon being loaded. Thus, in the practice of various embodiments of the invention, trehalose is transferred from a solution into the cells without degradation of the trehalose.

An extracellular medium of about 280-320 mOsm is considered iso-osmotic for cells, particularly erythrocytic cells, with regard to the amount of permeable solutes in the cytoplasm. Any increase of the amount of solutes in the extracellular medium creates an osmotic shock, ranging from a mild shock at about 350 mM trehalose to a strong shock at about 4200 mM trehalose, and a leakage of water which would reversibly reduce the cell volume. However, small molecular weight solutes, such as trehalose, in an extracellular medium in a concentration higher than about 320 mM, can pass through the membrane of a cell using a diffusion vector. It has been discovered that an extracellular

concentration of trehalose higher than about 450 mM (or mOsm), which is about 50% greater than an intracellular milliosmolarity, will produce an osmotic shock that will result in trehalose uptake. Increasing the extracellular trehalose concentration leads to even higher osmotic shock and higher trehalose uptake.

Molarity, or millimolarity, mM, is the number of moles (or millimoles) of a solute per liter of solution and is a measure of the concentration. Osmolarity (Osm), or milliosmolarity (mOsm), is a count of the number of dissolved particles per liter of solution and is a measure of the osmotic pressure exerted by solutes. Biological membranes, such as cell membranes, can be semi-permeable because they allow water and some small molecules to pass, but block the passage of proteins or macromolecules. Since the osmolarity of a solution is equal to the molarity times the number of particles per molecule, 600 mM trehalose is equal to 600 mOsm trehalose because trehalose does not dissociate in water. However, with respect to compounds that dissociate in water, such as NaCl, 1 mM NaCl is equal to 2 mOsm NaCl because it has two particles. Similarly, 100 mM NaCl is equal to 200 mOsm NaCl. Thus, for a 300 mOsm PBS buffer (100 mM NaCl, 9.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.6 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), 300 mOsm refers to all of the osmotically active particles in the PBS solution, with 200 mOsm of the 300 mOsm stemming from NaCl. A suitable PBS buffer for various embodiments of the present invention comprises 154 mM NaCl, 1.06 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.6 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4.

Other embodiments of the present invention provide for retaining a solute in a cell. Preferably, after the cells have been loaded with a solute, such as an oligosaccharide (e.g., trehalose), the cells are then washed. More preferably, during



the washing of the cells the solute is retained in the cells. The washing may be with a washing solution (e.g., such as a washing buffer having an oligosaccharide), and retention of the solute in the cell increases from about 25% to about 175% when a buffer concentration (e.g., the osmolarity of all osmotically active particles within the washing buffer solution) increases from about 50% to about 400%, more preferably from about 50% to about 150% when a buffer concentration increases from about 100% to about 300%, and most preferably from about 75% to about 125% (e.g., about 100%) when a buffer concentration increases from about 150% to about 250% (e.g., about 200%). The washing of the cell with a washing buffer includes employing a ratio of a buffer concentration (e.g., an extracellular buffer concentration) (mOsm) to an intracellular solute concentration (mM) ranging from about 14.0 to about 4.0, such as from about 12.0 to about 5.0, including from about 9.0 to about 6.0 and from about 8.0 to about 7.0 (e.g., about 7.5).

As indicated in patent application Serial No. 10/052,162, which claims the benefit of patent application Serial No. 09/501,773, filed February 10, 2000, with respect to common subject matter, the amount of the preferred trehalose loaded inside the cells ranges from about 10 mM to about 50 mM, and is achieved by incubating the cells to preserve biological properties during freeze-drying with a trehalose solution, preferably a trehalose solution that has up to about 50 mM trehalose therein. Higher concentrations of trehalose during incubation are not preferred, particularly since an embodiment of the invention includes preventing a decrease in a loading gradient, or a loading efficiency gradient, in the loading of the solute into the cell. It has been discovered that preventing a decrease in a loading gradient, or a loading efficiency gradient, in the loading of a oligosaccharide (i.e., trehalose)

into a cell comprises maintaining a concentration of the oligosaccharide in the oligosaccharide solution below a certain concentration (e.g., below a concentration ranging from about 35 mM to about 65 mM, more particularly below from about 40 mM to about 60 mM, or below from about 45 mM to about 55 mM, such as below about 50 mM). It has been further discovered that preventing a decrease in a loading gradient, or a loading efficiency gradient, in the loading of a oligosaccharide (i.e., trehalose) into a cell comprises maintaining a positive gradient of loading efficiency to concentration of the oligosaccharide in the oligosaccharide solution.

As further indicated in co-pending patent application Serial No. 10/052,162, the effective loading of trehalose is also accomplished by means of using an elevated temperature of from greater than about 25° C to less than about 40° C, more preferably from about 30°C to less than about 40°C, most preferably about 37°C. This is due to the discovery of the second phase transition for cells.

Referring now to Fig. 1, there is seen a graphical illustration from co-pending patent application Serial No. 10/052,162 of the loading efficiency of trehalose plotted versus incubation temperature of human platelets. The trehalose loading efficiency begins a steep slope increase at incubation temperatures above about 25°C and continues up to about 40°C. The trehalose concentration in the exterior solution (that is, the solute solution or loading buffer) and the temperature during incubation together lead to a trehalose uptake that occurs through fluid phase endocytosis. Example 1 below provides the more specific testing conditions and parameters which produced the graphical illustrations of Figure 1. It is believed

that the graphical illustration of the loading efficiency in Fig. 1 would be generally applicable for cells in general.

Referring now to Fig. 2, there is seen an illustration from co-pending patent application Serial No. 10/052,162 of trehalose loading efficiency for human blood platelets as a function of incubation time. More specifically, Figure 2 graphically illustrates the loading efficiency (cytosolic concentration divided by the extracellular concentration, the sum multiplied by 100) following incubation as a function of incubation time. Example 1 below provides the more specific testing conditions and parameters which produced the graphical illustrations of Figure 2. It is believed that the graphical illustration of the loading efficiency in Fig. 2 would also be generally applicable for cells in general.

Referring now to Figure 3, there is seen a graphical illustration from patent application Serial No. 10/052,162 of the internal trehalose concentration of human platelets versus external trehalose concentration as a function of 4<sup>0</sup> C and 37<sup>0</sup> C temperatures at a constant incubation or loading time. In Figure 4 there is seen a graphical illustration from patent application Serial No. 10/052,162 of the loading efficiency of trehalose into human platelets as a function of external trehalose concentration. Example 1 below provides the more specific testing conditions and parameters which produced the graphical illustrations of Figures 3 and 4. In additional embodiments of the present invention, it is further believed that the general findings illustrated in Figs. 3 and 4 with respect to platelets are generally broadly applicable to cells in general.

Thus, applying the findings illustrated in Fig. 3 and in Fig. 4 to solutes and cells in general, a decrease in a loading gradient or a loading efficiency gradient in the loading of a

solute into a cell may be prevented. For an embodiment of the present invention and as broadly illustrated in Figure 3, preventing a decrease in a loading gradient or a loading efficiency gradient in the loading of the solute (e.g., an oligosaccharide such as trehalose) into the cell comprises maintaining a concentration of the solute (e.g., an oligosaccharide such as trehalose) in the solute solution (e.g. an oligosaccharide solution such as a trehalose solution) below a solute concentration ranging from about 35 mM to about 65 mM, more specifically a solute concentration ranging from about 40 mM to about 60 mM, more specifically further a solute concentration ranging from about 45 mM to about 55 mM (e.g., about 50 mM). In another embodiment of the present invention and as best illustrated in Figure 4, preventing a decrease in a loading gradient or a loading efficiency gradient in the loading of the solute (e.g., an oligosaccharide, such as trehalose) into the cell comprises maintaining a positive gradient of loading efficiency (e.g., loading efficiency in %) to concentration (e.g., concentration in mM) of the solute in the solute solution (e.g. an oligosaccharide solution, such as a trehalose solution).

When a solute is loaded from a solute solution into one or more cells, the solute solution preferably has a solute concentration of sufficient magnitude to produce hyperosmotic pressure on the one or more cells. It has been discovered that the basis for the loading of the solute into the cells is dependent upon osmotic shock. The magnitude of osmotic shock and hyperosmotic pressure on the cells depends on the difference between internal solute concentration, or the intracellular osmolarity, within the cells, and the external solute concentration within the solute solution, or the extracellular

cellular solute concentration. For embodiments of the invention, the solute solution has a solute concentration ranging from about 320 mM to about 4000 mM, preferably from about 320 mM to about 2000 mM, more preferably from about 500 mM to about 1000 mM.

It has also been discovered that the basis for the loading of the solute into the cells is not only dependent upon osmotic shock, but is also dependent upon the thermal effects on flux of the solute across the membranes of the cells. The higher the thermal effects on flux of the solute across the membranes of the cells, the larger the amount of solute loaded into the cells. Stated alternatively, loading of a solute into cells increases as the temperature of the solute solution increases. Referring now to Figure 5, there is seen a graphical illustration of intracellular trehalose concentration as a function of extracellular trehalose concentration at respective temperatures of 4° C and 37° C. Thus, at a temperature ranging from about 30° C to about 40° C (e.g. at about 37° C) a gradient of a solute concentration (mM), such as an oligosaccharide (e.g., trehalose) concentration, within a cell (e.g., an erythrocytic cell) to extracellular solute concentration (mM) within a loading solution (or buffer) ranges from about 0.130 to about 0.200. At a temperature ranging from about 0° C to about 10° C (e.g. at about 4° C) a gradient of a solute concentration (mM), such as an oligosaccharide (e.g., trehalose) concentration, within a cell (e.g., an erythrocytic cell) to extracellular solute concentration (mM) within a loading solution (or buffer) ranges from about 0.04 to about 0.12, more specifically from about 0.04 to about 0.08, and from about 0.08 to about 0.12, depending on the quantity of extracellular solute concentration. Example 2 below provides the more specific

testing conditions and parameters which produced the graphical illustrations of Figure 5.

Referring now to Figure 6, there is seen a graphical illustration of the fragility index of erythrocytic cells incubated overnight at respective temperatures of 4° C and 37° C in the presence of and as a function of increasing extracellular trehalose concentrations. The osmotic fragility index was generated by the extent of hemolysis as a function of the NaCl concentration. The graphical illustration of Figure 6 represents a test for investigating the effects of hyperosmotic treatment rendering erythrocytic cells more sensitive to change in intracellular osmolarity. NaCl was loaded into erythrocytic cells from a 100 mOsm PBS buffer at loading 100 mOsm PBS buffer temperatures of 4° C and 37° C for extracellular trehalose concentrations of 0 mM (control cells), 250 mM, 500 mM, 600 mM, 700 mM, 800 mM and 1000 mM. Data blocks, respectively generally indicated as **60** and **62**, represent the intracellular trehalose concentrations for 100 mOsm PBS solution loading temperatures of 4° C and 37° C. The mOsm/kg values of NaCl represent extracellular NaCl osmolarity of the erythrocytic cells resulting from the transfer of NaCl from the PBS loading buffer into the erythrocytic cells. The erythrocytic cells that had been loaded in trehalose solutions (between 250 mM and 1000 mM) in 100 mOsm PBS were suspended in increasing concentrations of NaCl (between 50 and 600 mOsm NaCl). The percent hemolysis measured after resuspending the loaded cells in NaCl represents the fragility index. The data show that the erythrocytic cells were stable osmotically in trehalose media with concentrations between 250 mM and 800 mM trehalose at both 37° C and 4° C. In 1000 mM trehalose at 37° C, there is a high increase in the fragility index suggesting that the cells were unstable in this

medium (1000mM trehalose in 100 mOsm PBS). Clearly, at moderate intracellular concentrations of trehalose, osmotic fragility as measured by a standard assay was not severely altered. Thus, erythrocytic cells may be loaded with trehalose concentrations up to about 900 mM (i.e., a trehalose concentration between 800 mM and 1000 mM). Example 3 below provides specific testing conditions and parameters which produced the graphical illustrations of Figure 6.

Thus, from the findings graphically illustrated in Figs. 5 and 6, and as more fully explained in Examples 2 and 3 below, temperature of a solute loading solution has an effect in loading a solute from a solute solution into a cell. The effects of temperature, as well as cellular hemolysis, of a trehalose loading solution in loading of trehalose into a cell was tested. The test results are illustrated in Figure 7, which is a graphical illustration of trehalose uptake (i.e., intracellular trehalose mM) and hemolysis (i.e., % hemolysis) as a function of incubation temperature ( $^{\circ}\text{C}$ ). Figure 7 illustrates that effective loading occurs above  $30^{\circ}\text{C}$ , and that as the loading temperature of the trehalose loading solution increases, there is slight hemolysis. Example 4 below provides the more specific testing conditions and parameters which produced the graphical illustrations of Figure 7.

As previously indicated, after a cell (e.g., an erythrocytic cell) has been loaded with a solute (e.g., trehalose), further embodiments of the present invention provide for retaining the solute in the cells. One means for retaining solute within solute-loaded cells is to wash the cells, more specifically by washing the cells and retaining the solute in the cells during the washing. As also previously indicated, the washing of the cells is preferably with a washing buffer. It

has been discovered that retention of the solute in the cells increases from about 25% to about 175% when a buffer concentration (e.g., the osmolarity of all osmotically active particles within the washing buffer solution) increases from about 50% to about 400%, more preferably from about 50% to about 150% when a buffer concentration increases from about 100% to about 300%, and most preferably from about 75% to about 125% (e.g., about 100%) when a buffer concentration increases from about 150% to about 250% (e.g., about 200%). It has been further discovered that the washing of the cells with a washing buffer includes employing a ratio of an extracellular buffer concentration (mOsm) to an intracellular trehalose concentration (mM) ranging from about 14.0 to about 4.0, more particularly from about 12.0 to about 5.0, including from about 9.0 to about 6.0 and from about 8.0 to about 7.0 (e.g., about 7.5). Thus, because solute loaded cells are hyperosmotic to a washing buffer, increasing the extracellular osmolarity increases retention of the solute during washing of the cells, as shown in Figure 8 which graphically illustrates trehalose uptake (i.e., intracellular trehalose mM) as a function of the osmolarity of the washing buffer. As shown in Figure 8, when the extracellular buffer concentration was increased from 300 mOsm PBS to 900 mOsm PBS during washing, the final intracellular trehalose concentration doubled. The cells that were washed with 300 mOsm PBS had a 65 mM trehalose concentration, whereas the cells that were washed with 900 mOsm PBS had a 115 mM intracellular trehalose concentration. Example 5 below provides the more specific testing conditions and parameters which produced the graphical illustrations of Figure 8.

After the cells have been effectively loaded with a solute and subsequently washed, the cells may then be contacted with a drying buffer. The drying buffer should include the solute,



preferably in amounts up to about 100 mM. The solute in the drying buffer assists in spatially separating the cells as well as stabilizing the cell membranes on the exterior. The drying buffer preferably also includes a bulking agent (to further separate the cells). Albumin may serve as a bulking agent, but other polymers may be used with the same effect. If albumin is used, it is preferably from the same species as the cells. Suitable other polymers, for example, are water-soluble polymers such as HES (hydroxy ethyl starch) and dextran.

The solute loaded cells in the drying buffer may then be dried. Preferably, the solute loaded cells are dried while simultaneously cooling to a temperature below about  $-32^{\circ}\text{C}$ . A cooling, that is, freezing, rate is preferably between  $-30^{\circ}\text{C}$  and  $-1^{\circ}\text{C}/\text{min}$ . and more preferably between about  $-2^{\circ}\text{C}/\text{min}$  to  $-5^{\circ}\text{C}/\text{min}$ . Drying may be continued until about a residual water content of about 3% is achieved. During the initial stages of lyophilization, the pressure is preferably at about  $10 \times 10^{-6}$  torr. As the samples dry, the temperature can be raised to be warmer than  $-32^{\circ}\text{C}$ . Based upon the bulk of the sample, the temperature and the pressure it can be empirically determined what the most efficient temperature values should be in order to maximize the evaporative water loss. Dried cell compositions preferably have less than about 5 weight percent water.

After drying and storage of the cells, the process of using such a dehydrated cell composition comprises rehydrating the cells. The rehydration preferably includes a prehydration step, sufficient to bring the water content of the freeze-dried cells to between about 20 weight percent and about 50 percent, preferably from about 20 weight percent to about 40 weight percent. More preferably, when reconstitution of the freeze dried cells is desired, the freeze dried cells are prehydrated

in moisture saturated air at about 37°C for about five minutes to about three hours, followed by rehydration. Rehydration of the prehydrated cells may be with any aqueous based solutions, depending upon the intended application.

Embodiments of the present invention will be illustrated by the following set forth examples which are being given to set forth the presently known best mode and by way of illustration only and not by way of any limitation. It is to be understood that all materials, chemical compositions and procedures referred to below, but not explained, are well documented in published literature and known to those artisans possessing skill in the art. All materials and chemical compositions whose source(s) are not stated below are readily available from commercial suppliers, who are also known to those artisans possessing skill in the art. All parameters such as concentrations, mixing proportions, temperatures, rates, compounds, etc., submitted in these examples are not to be construed to unduly limit the scope of the invention. Abbreviations used in the examples, and elsewhere, are as follows:

DMSO = dimethylsulfoxide

ADP = adenosine diphosphate

PGE1 = prostaglandin E1

HES = hydroxy ethyl starch

FTIR = Fourier transform infrared spectroscopy

EGTA = ethylene glycol-bis(2-aminoethyl ether) N,N,N',N',  
tetra-acetic acid

TES = N-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic  
acid

HEPES = N-(2-hydroxyl ethyl) piperazine-N'-(2-ethanesulfonic acid)

PBS = phosphate buffered saline

HSA = human serum albumin

BSA = bovine serum albumin

ACD = citric acid, citrate, and dextrose

M $\beta$ CD = methyl- $\beta$ -cyclodextrin

#### EXAMPLE 1

Washing of Platelets. Platelet concentrations were obtained from the Sacramento blood center or from volunteers in our laboratory. Platelet rich plasma was centrifuged for 8 minutes at 320 x g to remove erythrocytes and leukocytes. The supernatant was pelleted and washed two times (480 x g for 22 minutes, 480 x g for 15 minutes) in buffer A (100 mM NaCl, 10 mM KCl, 10 mM EGTA, 10 mM imidazole, pH 6.8). Platelet counts were obtained on a Coulter counter T890 (Coulter, Inc., Miami, Florida).

Loading of Lucifer Yellow CH into Platelets. A fluorescent dye, lucifer yellow CH (LYCH), was used as a marker for penetration of the membrane by a solute. Washed platelets in a concentration of  $1-2 \times 10^9$  platelets/ml were incubated at various temperatures in the presence of 1-20 mg/ml LYCH. Incubation temperatures and incubation times were chosen as indicated. After incubation the platelets suspensions were spun down for 20 s at 14,000 RPM (table centrifuge), resuspended in buffer A, spun down for 20 s in buffer A and resuspended. Platelet counts were obtained on a Coulter counter and the samples were pelleted

(centrifugation for 45 s 25 at 14,000 RPM, table centrifuge). The pellet was lysed in 0.1% Triton buffer (10 mM TES, 50 mM KCl, pH 6.8). The fluorescence of the lysate was measured on a Perkin-Elmer LSS spectrofluorimeter with excitation at 428 nm (SW 10 nm) and emission at 530 nm (SW 10 nm). Uptake was calculated for each sample as nanograms of LYCH per cell using a standard curve of LYCH in lysate buffer. Standard curves of LYCH, were found to be linear up to 2000 run  $\text{ml}^{-1}$ .

Visualization of cell-associated Lucifer Yellow. LYCH loaded platelets were viewed on a fluorescence microscope (Zeiss) employing a fluorescein filter set for fluorescence microscopy. Platelets were studied either directly after incubation or after fixation with 1% paraformaldehyde in buffer. Fixed cells were settled on poly-L-lysine coated cover slides and mounted in glycerol.

Loading of Platelets with Trehalose. Washed platelets in a concentration of  $1-2 \times 10^9$  platelets/ml were incubated at various temperatures in the presence of 1-20 mg/ml trehalose. Incubation temperatures were chosen from 4°C to 37°C. Incubation times were varied from 0.5 to 4 hours. After incubation the platelet solutions were washed in buffer A two times (by centrifugation at 14,000 RPM for 20 s in a table centrifuge). Platelet counts were obtained on a coulter counter. Platelets were pelleted (45 s at 14,000 RPM) and sugars were extracted from the pellet using 80% methanol. The samples were heated for 30 minutes at 80°C. The methanol was 10 evaporated with nitrogen, and the samples were kept dry and redissolved in  $\text{H}_2\text{O}$  prior to analysis. The amount of trehalose in the platelets was quantified using the anthrone reaction (Umbreit et al., *Manometric and Biochemical Techniques*, 5th Edition, 1972). Samples were redissolved in 3 ml  $\text{H}_2\text{O}$  and 6 ml anthrone reagents (2 g anthrone dissolved in 10M

sulfuric acid). After vortex mixing, the samples were placed in a boiling water bath for 3 minutes. Then the samples were cooled on ice and the absorbance was measured at 620 nm on a Perkin Elmer spectrophotometer. The amount of platelet associated trehalose was determined using a standard curve of trehalose. Standard curves of trehalose were found to be linear from 6 to 300  $\mu\text{g}$  trehalose per test tube.

Quantification of Trehalose and LYCH Concentration. Uptake was calculated for each sample as micrograms of trehalose or LYCH per platelet. The internal trehalose concentration was calculated assuming a platelet radius of 1.2  $\mu\text{m}$  and by assuming that 50% of the platelet volume is taken up by the cytosol (rest is membranes). The loading efficiency was determined from the cytosolic trehalose or LYCH concentration and the concentration in the loading buffer.

Fig. 1 shows the effect of temperature on the loading efficiency of trehalose into human platelets after a 4 hour incubation period with 50 mM external trehalose. The effect of the temperature on the trehalose uptake showed a similar trend as the LYCH uptake. The trehalose uptake is relatively low at temperatures of 22°C and below (below 5%), but at 37°C the loading efficiency of trehalose is 35% after 4 hours.

When the time course of trehalose uptake is studied at 37°C, a biphasic curve can be seen (Fig. 2). The trehalose uptake is initially slow ( $2.8 \times 10^{-11}$  mol/m<sup>2</sup>s from 0 to 2 hours), but after 2 hours a rapid linear uptake of  $3.3 \times 10^{-10}$  mol/m<sup>2</sup>s can be observed. The loading efficiency increases up to 61% after an incubation period of 4 hours. This high loading efficiency is a strong indication that the trehalose is homogeneously distributed in the platelets rather than located in pinocytosed vesicles.

The uptake of trehalose as a function of the external trehalose concentration is shown in Fig. 3, which graphically illustrates the internal trehalose concentration of human platelets versus external trehalose concentration as a function of temperature at a constant incubation or loading time. The uptake of trehalose is linear in the range from 0 to 30 mM external trehalose. The highest internal trehalose concentration is obtained with 50 mM external trehalose. At higher concentrations than 50 mM the internal trehalose concentration decreases again. Even when the loading buffer at these high trehalose concentrations is corrected for isotonicity by adjusting the salt concentration, the loading efficiency remains low. Platelets become swollen after 4 hours incubation in 75 mM trehalose. Figure 4 graphically illustrates the loading efficiency of trehalose into human platelets as a function of external trehalose concentration.

The stability of the platelets during a 4 hours incubation period was studied using microscopy and flow cytometric analysis. No morphological changes were observed after 4 hours incubation of platelets at 37°C in the presence of 25 mM external trehalose. Flow cytometric analysis of the platelets showed that the platelet population is very stable during 4 hours incubation. No signs of microvesicle formation could be observed after 4 hours incubation, as can be judged by the stable relative proportion of microvesicle gated cells (less than 3%). The formation of microvesicles is usually considered as the first sign of platelet activation (Owners et al., *Trans. Med. Rev.*, 8, 27-44, 1994). Characteristic antigens of platelet activation include: glycoprotein 53 (gp53, a lysosomal membrane marker), PECAM-1 (platelet endothelial cell adhesion molecule-1,

an alpha granule constituent), and P-selection (an alpha granule membrane protein).

Figure 5 graphically illustrates the loading efficiency of trehalose into human erythrocytic cells as a function of external trehalose concentration at respective temperatures of 4° C and 37° C. Erythrocytic cells were exposed to trehalose for 18 hours at either 4° C or 37° C. The trehalose concentration in the incubation medium varied between 230 mM and 1000 mM. Each incubation buffer contained trehalose (between 230 mM and 1000 mM) and 100 mOsm PBS pH 7.2. Increase in the trehalose concentration in the loading medium results in an increase in the sugar uptake, reaching about 100 mM cytoplasmic trehalose in erythrocytes incubated in 1000 mM trehalose and 100 mOsm PBS. At 4° C, the uptake was very limited, being about 25 mM. The trehalose intake was measured using anthrone assay and confirmed by high performance liquid chromatography. It is clear that there was substantial loading at 37° C, but not at 4° C. Furthermore, trehalose loading was not significant unless the extracellular cellular trehalose concentration gave a hyperosmotic pressure. Since intracellular osmolarity for erythrocytic cells is about 300 mOsm, it is clear that raising the extracellular osmolarity was required for more effective loading of trehalose.

#### EXAMPLE 2

Figure 5 graphically illustrates the loading efficiency of trehalose into human erythrocytic cells as a function of external trehalose concentration at respective temperatures of 4° C and 37° C. Erythrocytic cells were exposed to trehalose for 18 hours at either 4° C or 37° C. The trehalose

concentration in the incubation medium varied between 230 mM and 1000 mM. Each incubation buffer contained trehalose (between 230 mM and 1000 mM) and 100 mOsm PBS pH 7.2. Increase in the trehalose concentration in the loading medium results in an increase in the sugar uptake, reaching about 100 mM cytoplasmic trehalose in erythrocytes incubated in 1000 mM trehalose and 100 mOsm PBS. At 4° C, the uptake was very limited, being about 25 mM. The trehalose intake was measured using anthrone assay and confirmed by high performance liquid chromatography. It is clear that there was substantial loading at 37° C, but not at 4° C. Furthermore, trehalose loading was not significant unless the extracellular cellular trehalose concentration gave a hyperosmotic pressure. Since intracellular osmolarity for erythrocytic cells is about 300 mOsm, it is clear that raising the extracellular osmolarity was required for more effective loading of trehalose.

### EXAMPLE 3

Figure 6 graphically illustrates the fragility index of erythrocytic cells incubated overnight at respective temperatures of 4° C and 37° C in the presence of and as a function of increasing intracellular trehalose concentrations. The osmotic fragility index was generated by the extent of hemolysis as a function of the NaCl concentration. The erythrocytic cells that had been loaded in trehalose solutions (between 250 mM and 1000 mM) in 100 mOsm PBS were suspended in increasing concentrations of NaCl (between 50 and 600 mOsm NaCl). The percent hemolysis measured after resuspending the loaded cells in NaCl represents the fragility index. The data show that the erythrocytic cells were stable osmotically in trehalose media with concentrations between 250 mM and 800 mM



trehalose at both 37° C and 4° C. In 1000 mM trehalose at 37° C, there is a high increase in the fragility index suggesting that the cells were unstable in this medium (1000mM trehalose in 100 mOsm PBS).

#### EXAMPLE 4

Figure 7 graphically illustrates trehalose uptake (i.e., intracellular trehalose mM) and hemolysis (i.e., % hemolysis) as a function of incubation temperature (°C). The incubation temperature was varied between 4° C and 37° C. The erythrocytic cells were incubated for 6 hours in 800 mM trehalose in 100 mOsm PBS pH 7.2. Between 4° C and 30° C, the cytoplasmic trehalose was very low (between 1 and 4 mM). It was considerably increased (up to 35 mM cytoplasmic trehalose) during 6 hours incubation at 37° C.

#### EXAMPLE 5

Figure 8 graphically illustrates intracellular trehalose (mM) as a function of the osmolarity of the washing buffer. Earlier morphological data showed that along with biconcave discoid erythrocytic cells, there is about 20% of cells with modified shape (spherocytes and schistocytes). The issue was what was the loading capacity of these cells and how much they contribute to the amount of trehalose that was to be detected. This issue was investigated by washing the trehalose loaded erythrocytic cells (loaded at 35° C for 16 hours in 800 mM trehalose in 100 mOsm PBS pH 7.2) in buffers with different osmolarity (300 mOsm PBS or 900 mOsm PBS) and estimating the cytoplasmic sugar concentration. The loaded cells were washed

with either 300 mOsm PBS pH 7.2 (which is the isotonic medium for erythrocytic cells) or 900 mOsm PBS pH 7.2 (which matches the tonicity of the loading medium). The data in Figure 8 illustrated that there is a decrease in the intracellular sugar concentration suggesting that a fraction of the cells was lost during the washing procedure.

### Conclusion

Embodiments of the present invention provide that trehalose, a sugar found at high concentrations in organisms that normally survive dehydration, may be used to preserve biological structures in the dry state. Cells may be loaded with trehalose under the previously specified conditions, and the loaded cells can be dried with excellent recovery.

While the present invention has been described herein with reference to particular embodiments thereof, a latitude of modification, various changes and substitutions are intended in the foregoing disclosure, and it will be appreciated that in some instances some features of the invention will be employed without a corresponding use of other features without departing from the scope and spirit of the invention as set forth. Therefore, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from the essential scope and spirit of the present invention. It is intended that the invention not be limited to the particular embodiment disclosed as the best mode contemplated for carrying out this invention, but that the invention will include all embodiments and equivalents falling within the scope of the appended claims.

**What Is Claimed Is:**

1. A method for loading a solute into a cell comprising:

disposing a cell in a solution having a solute concentration of sufficient magnitude to produce hyperosmotic pressure on the cell for transferring a solute from the solution into the cell.

2. The method of Claim 1 wherein said solute concentration includes an extracellular cellular solute concentration for elevating extracellular osmolarity within the solution to a value which is greater than a value of the intracellular osmolarity of the cell.

3. The method of Claim 1 wherein said transferring a solute is by fluid phase endocytosis.

4. The method of Claim 1 wherein said solute comprises trehalose and said cell comprises an erythrocytic cell.

5. The method of Claim 4 wherein said transferring of trehalose from the solution into the erythrocytic cell is without degradation of the trehalose.

6. The method of Claim 4 wherein a gradient of trehalose concentration (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the solution ranges from about 0.130 to about 0.200.

7. The method of Claim 4 wherein a gradient of trehalose concentration (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the solution ranges from about 0.04 to about 0.12.
8. The method of Claim 4 wherein a gradient of trehalose concentration (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the solution ranges from about 0.08 to about 0.12.
9. The method of Claim 4 wherein said solute solution has a trehalose concentration ranging from about 320 mM to about 4000 mM.
10. The method of Claim 4 wherein said solute solution has a trehalose concentration ranging from about 320 mM to about 2000 mM.
11. The method of Claim 4 wherein said solute solution has a trehalose concentration ranging from about 500 mM to about 1000 mM.
12. A cell produced in accordance with the method of Claim 1.
13. An erythrocytic cell produced in accordance with the method of Claim 11.
14. A method for loading trehalose into an erythrocytic cell comprising disposing an erythrocytic cell in a trehalose solution having a trehalose concentration of at least about 25 % greater than the intracellular osmolarity of the erythrocytic cell for loading the trehalose into the erythrocytic cell.

15. The method of Claim 14 wherein said loading the trehalose into the erythrocytic cell is by fluid phase endocytosis.

16. The method of Claim 14 wherein said loading of the trehalose from the trehalose solution into the erythrocytic cell is without degradation of the trehalose.

17. The method of Claim 14 said loading of the trehalose produces a loaded erythrocytic cell having a gradient of loaded trehalose concentration (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the trehalose solution ranging from about 0.130 to about 0.200.

18. The method of Claim 14 wherein said loading of the trehalose produces a loaded erythrocytic cell having a gradient of loaded trehalose concentration (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the trehalose solution ranging from about 0.04 to about 0.08.

19. The method of Claim 14 wherein said loading of the trehalose produces a loaded erythrocytic cell having a gradient of loaded trehalose concentration (mM) within the erythrocytic cell to extracellular trehalose concentration (mM) within the trehalose solution ranging from about 0.04 to about 0.12.

20. The method of Claim 14 wherein said trehalose solution has a trehalose concentration of at least about 50% greater than the intracellular osmolarity of the erythrocytic cell.

21. The method of Claim 14 wherein said trehalose solution has a trehalose concentration ranging from about 25 % to at least

about 1000% greater than the intracellular osmolarity of the erythrocytic cell.

22. An erythrocytic cell produced in accordance with the method of Claim 14.

23. The method of Claim 1 additionally comprising

preventing a decrease in a loading efficiency gradient in the loading of the solute into the cell.

24. The method of Claim 23 wherein said solute comprises an oligosaccharide and said preventing a decrease in a loading efficiency gradient in the loading of the oligosaccharide into the cell comprises maintaining a concentration of the oligosaccharide in the oligosaccharide solution below a concentration ranging from about 35 mM to about 65 mM.

25. The method of Claim 23 wherein said loading comprises loading by fluid phase endocytosis.

26. The method of Claim 24 wherein said loading comprises loading by fluid phase endocytosis.

27. The method of Claim 23 wherein said solute comprises an oligosaccharide and said preventing a decrease in a loading efficiency gradient in the loading of the oligosaccharide into the cell comprises maintaining a positive gradient of loading efficiency to concentration of the oligosaccharide in the oligosaccharide solution.

28. The method of Claim 23 wherein said solute comprises an oligosaccharide and said preventing a decrease in a loading efficiency gradient in the loading of the oligosaccharide into the cell comprises maintaining a positive gradient of loading efficiency (%) to concentration (mM) of the oligosaccharide in the oligosaccharide solution.

29. The method of Claim 27 wherein said oligosaccharide comprises trehalose.

30. The method of Claim 28 wherein said oligosaccharide comprises trehalose.

31. A method for loading trehalose into cells comprising:

disposing cells in a trehalose solution having a trehalose concentration of at least about 25 % greater than the intracellular osmolarity of the cells for loading trehalose into the cells; and

preventing a decrease in a loading efficiency gradient in the loading of the trehalose into the cells.

32. The method of Claim 31 wherein said preventing a decrease in a loading efficiency gradient in the loading of the trehalose into the cells comprises maintaining a concentration of the trehalose in the trehalose solution below a concentration ranging from about 35 mM to about 65 mM.

33. The method of Claim 31 wherein said loading comprises loading by fluid phase endocytosis.

34. The method of Claim 32 wherein said loading comprises loading by fluid phase endocytosis.

35. The method of Claim 31 wherein said preventing a decrease in a loading efficiency gradient in the loading of the trehalose into the cells comprises maintaining a positive gradient of loading efficiency to concentration of the trehalose in the trehalose solution.

36. The method of Claim 31 wherein said preventing a decrease in a loading efficiency gradient in the loading of the trehalose into the cells comprises maintaining a positive gradient of loading efficiency (%) to concentration (mM) of the trehalose in the trehalose solution.

37. The method of Claim 31 wherein said cells comprise erythrocytic cells.

38. The method of Claim 36 wherein said cells comprise erythrocytic cells.

39. A method for loading an oligosaccharide into cells comprising:

disposing cells in an oligosaccharide solution having an oligosaccharide concentration of at least about 25 % greater than the intracellular osmolarity of the cells for loading oligosaccharide into the cells; and

preventing a decrease in a loading gradient in the loading of the oligosaccharide into the cells.



40. The method of Claim 39 wherein said preventing a decrease in a loading gradient in the loading of the oligosaccharide into the cells comprises maintaining a concentration of the oligosaccharide in the oligosaccharide solution below a concentration ranging from about 35 mM to about 65 mM.

41. The method of Claim 39 wherein said loading comprises loading by fluid phase endocytosis.

42. The method of Claim 40 wherein said loading comprises loading by fluid phase endocytosis.

43. The method of Claim 39 wherein said preventing a decrease in a loading gradient in the loading of the oligosaccharide into the cells comprises maintaining a positive gradient of concentration of oligosaccharide loaded into the cells to concentration of the oligosaccharide in the oligosaccharide solution.

44. The method of Claim 43 wherein said oligosaccharide comprises trehalose.

45. The method of Claim 39 wherein said cells comprise erythrocytic cells.

46. The method of Claim 1 additionally comprising retaining the solute in the cell.

47. The method of Claim 1 additionally comprising washing the cell, and retaining the solute in the cell during the washing.

48. The method of Claim 47 wherein said washing is with a washing buffer, and retention of the solute in the cell increases from about 25% to about 175% when a buffer concentration increases from about 50% to about 400%.

49. The method of Claim 47 wherein said washing is with a washing buffer, and retention of the solute in the cell increases from about 50% to about 150% when a buffer concentration increases from about 100% to about 300%.

50. The method of Claim 47 wherein said washing is with a washing buffer, and retention of the solute in the cell increases from about 75% to about 125% when a buffer concentration increases from about 150% to about 250%.

51. The method of Claim 47 wherein said washing is with a washing buffer, and retention of the solute in the cell increases about 100% when a buffer concentration increases about 200%.

52. The method of Claim 1 additionally comprising washing the cell with a washing buffer wherein a ratio of an extracellular buffer concentration (mOsm) to an intracellular solute concentration (mM) ranges from about 14.0 to about 4.0.

53. The method of Claim 1 additionally comprising washing the cell with a washing buffer wherein a ratio of an extracellular buffer concentration (mOsm) to an intracellular solute concentration (mM) ranges from about 12.0 to about 5.0.

54. The method of Claim 1 additionally comprising washing the cell with a washing buffer wherein a ratio of an extracellular

buffer concentration (mOsm) to an intracellular solute concentration (mM) ranges from about 9.0 to about 6.0.

55. The method of Claim 1 additionally comprising washing the cell with a washing buffer wherein a ratio of an extracellular buffer concentration (mOsm) to an intracellular solute concentration (mM) ranges from about 8.0 to about 7.0.

56. The method of Claim 14 additionally comprising retaining the trehalose in the erythrocytic cell.

57. The method of Claim 14 additionally comprising washing the erythrocytic cell, and retaining the trehalose in the erythrocytic cell during the washing.

58. The method of Claim 57 wherein said washing is with a washing buffer, and retention of the trehalose in the erythrocytic cell increases from about 25% to about 175% when a buffer concentration increases from about 50% to about 400%.

59. The method of Claim 47 wherein said washing is with a washing buffer, and retention of the trehalose in the erythrocytic cell increases from about 50% to about 150% when a buffer concentration increases from about 100% to about 300%.

60. The method of Claim 57 wherein said washing is with a washing buffer, and retention of the trehalose in the erythrocytic cell increases from about 75% to about 125% when a buffer concentration increases from about 150% to about 250%.

61. The method of Claim 57 wherein said washing is with a washing buffer, and retention of the trehalose in the

erythrocytic cell increases about 100% when a buffer concentration increases about 200%.

62. The method of Claim 14 additionally comprising washing the erythrocytic cell with a washing buffer wherein a ratio of an extracellular buffer concentration (mOsm) to an intracellular trehalose concentration (mM) ranges from about 14.0 to about 4.0.

63. The method of Claim 14 additionally comprising washing the erythrocytic cell with a washing buffer wherein a ratio of an extracellular buffer concentration (mOsm) to an intracellular trehalose concentration (mM) ranges from about 12.0 to about 5.0.

64. The method of Claim 14 additionally comprising washing the erythrocytic cell with a washing buffer wherein a ratio of an extracellular buffer concentration (mOsm) to an intracellular trehalose concentration (mM) ranges from about 9.0 to about 6.0.

65. The method of Claim 14 additionally comprising washing the erythrocytic cell with a washing buffer wherein a ratio of an extracellular buffer concentration (mOsm) to an intracellular trehalose concentration (mM) ranges from about 8.0 to about 7.0.

66. A method for retaining a solute in a cell comprising disposing a cell containing a solute in a solution wherein a ratio of an extracellular buffer concentration (mOsm) to an intracellular solute concentration (mM) ranges from about 14.0 to about 4.0.

Applicant: John H. Crowe et al.  
Serial No.: Unknown  
Filed: August 6, 2003  
Title: ERYTHROCYTIC CELLS AND METHOD FOR LOADING SOLUTES

Group: Unknown  
Examiner: Unknown  
Docket No. 010023-000510

**COMBINED DECLARATION AND POWER OF ATTORNEY**  
**[ORIGINAL, DESIGN, SUPPLEMENTAL, NATIONAL STAGE OF PCT, DIVISIONAL, CONTINUATION OR**  
**CONTINUATION-IN-PART]**

---

As a below named inventor, I hereby declare that:

**TYPE OF DECLARATION**

This declaration is of the following type:

- ☒ original (regular)
- ☐ design
- ☐ supplemental-continuation of PCT Application in the U.S.

NOTE: If the declaration is for an International Application being filed as a divisional; continuation, or continuation-in-part, do not check the next item; check appropriate one of last three items.

- ☐ national stage of PCT divisional continuation continuation-in-part

**INVENTORSHIP IDENTIFICATION**

WARNING: If the inventors are each not the inventors of all of the claims, an explanation of the facts, including the ownership of all the claims at the time the last claimed invention was made, should be submitted.

My residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention described in

**SPECIFICATION IDENTIFICATION**

- ☒ the specification filed herewith by the above-named inventors, with the title listed above.
- ☐ the specification filed herewith by the above-named inventors, with the title listed above, and which was amended by the Preliminary Amendment filed herewith.
- ☐ the specification identified above, as amended by the Preliminary Amendment filed herewith.
- ☐ the specification identified above, Serial No. \_\_\_\_\_ filed on \_\_\_\_\_.
- ☐ PCT International Application No. \_\_\_\_\_ filed on \_\_\_\_\_, and amended under PCT Article 19 on \_\_\_\_\_ (if applicable).

**ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR**

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. §1.56.

**PRIORITY CLAIM (35 U.S.C. § 119(a)-(d))**

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or under 35 U.S.C. § 365(b) of any PCT international application(s) designating at least one country other than the United States of America listed below, and have also identified below any foreign application(s) or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

☒ No such applications have been filed.

☐ Such applications have been filed as follows:

**PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS APPLICATION AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. § 119(a)-(d)**

| COUNTRY (or indicate if PCT) | APPLICATION NUMBER | FILING DATE | PRIORITY CLAIMED UNDER 37 U.S.C. § 119 |                             |
|------------------------------|--------------------|-------------|--|-----------------------------|
|                              |                    |             | <input type="checkbox"/> YES           | <input type="checkbox"/> NO |
|                              |                    |             | <input type="checkbox"/> YES           | <input type="checkbox"/> NO |
|                              |                    |             | <input type="checkbox"/> YES           | <input type="checkbox"/> NO |
|                              |                    |             | <input type="checkbox"/> YES           | <input type="checkbox"/> NO |

**CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S) (35 U.S.C. § 119(e))**

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional applications(s) listed below:

| PROVISIONAL APPLICATION SERIAL NUMBER | FILING DATE OF PROVISIONAL APPLICATION |
|---------------------------------------|--|
|                                       |  |
|                                       |  |
|                                       |  |
|                                       |  |

**CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S) UNDER 35 U.S.C. § 120**

(Complete this part only if this is a divisional, continuation, CIP or national stage of PCT.)

I/We hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability under 37 C.F.R. § 1.56, which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

**PRIOR U.S. PROVISIONAL APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. § 120:**

**1. U.S. Applications:**

SERIAL NUMBER

FILING DATE

|  |  |
|--|--|
|  |  |
|  |  |
|  |  |
|  |  |

**2. PCT Applications Designating The U.S.**

PCT APPLICATION NUMBER

PCT FILING DATE

U.S. SERIAL NUMBER

|  |  |  |
|--|--|--|
|  |  |  |
|  |  |  |
|  |  |  |
|  |  |  |

**POWER OF ATTORNEY**

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the United States Patent and Trademark Office connected therewith:

John W. Carpenter

26,447

Charles J. Kulas

35,809

- ☐ Attached as part of this declaration and power of attorney is the authorization of the above-named attorney to accept and follow instructions from my representative(s).

**SEND CORRESPONDENCE TO:**

**DIRECT TELEPHONE CALLS TO:**

*Carpenter & Kulas, LLP  
1900 Embarcadero Road, Suite 109  
Palo Alto, CA 94303*

*John W. Carpenter  
(650) 842-0303*

**DECLARATION**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

**SIGNATURE(S)**

**Full name of first inventor: John H. Crowe**

Inventor's

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Country of Citizenship: USA

Residence: 1111 Cottonwood Court, Davis, CA 95616

P.O. Address: same

Full name of second inventor: Fern Tablin

Inventor's

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Country of Citizenship: USA

Residence: 608 Huble Street, Davis, CA 95616

P.O. Address: same

Full name of third inventor: Nelly M. Tsvetkova

Inventor's

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Country of Citizenship: Bulgarian

Residence: 3114 Newport Terrace, Davis, CA 95616

P.O. Address: same

Full name of fourth inventor: Zsolt Torok

Inventor's

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Country of Citizenship: Hungary

Residence: 4501 Alhambra Drive, #228, Davis, CA 95616

P.O. Address: same

Full name of fifth inventor: Gyana R. Satpathy

Inventor's

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Country of Citizenship: India

Residence: 424 Russell Park #2, Davis, CA 95616

P.O. Address: same

Full name of sixth inventor: Denis M. Dwyre

Inventor's

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Country of Citizenship: USA

Residence: 134 Ashford Place, Iowa City, IA 52245

P.O. Address: same



CHECK PROPER BOX(ES) FOR ANY OF THE FOLLOWING ADDED PAGE(S) WHICH FORM A PART OF THIS DECLARATION

- ☒ Signature for third and subsequent joint inventors.  
Number of pages added 1
- ☐ Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor.  
Number of pages added \_\_\_\_\_.
- ☐ Signature for inventor who refuses to sign or cannot be reached by person authorized under 37 CFR 1.47.  
Number of pages added \_\_\_\_\_.
- ☐ Signature by one joint inventor on behalf of deceased inventor(s) where legal representative cannot be appointed in time under 37 CFR 1.47.  
Number of pages added \_\_\_\_\_.
- ☐ Authorization of attorney(s) to accept and follow instructions from representative.

☒ This Declaration ends with this page.

**Abstract of the Disclosure**

A dehydrated composition is provided that includes freeze-dried erythrocytic cells. A method for loading a solute into a cell comprising disposing a cell in a solution having a solute concentration of sufficient magnitude to produce hyperosmotic pressure on the cell for transferring a solute from the solution into the cell. A method for retaining a solute in a cell.

Please type a plus sign (+) inside this box → ☐

PTO/SB/05 (03-01)

Approved for use through 10/31/2002. OMB 0651-0032

U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

# UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. 1.53(b))

Attorney Docket No. 010023-005100US

First Inventor John H. Crowe, et al.

Title ERYTHROCYTIC CELLS AND METHOD FOR LOADING SOLUTES

Express Mail Label No. EU121998647US

## APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ Fee Transmittal Form (e.g., PTO/SB/17)  
(Submit an original and a duplicate for fee processing)
2. ☒ Applicant claims small entity status.  
See 37 CFR 1.27.
3. ☒ Specification (Total Pages )  
(preferred arrangement set forth below)
  - Descriptive title of the Invention
  - Cross Reference to Related Applications
  - Statement Regarding Fed sponsored R & D
  - Reference to sequence listing, a table, or a computer program listing appendix
  - Background of the Invention
  - Brief Summary of the Invention
  - Brief Description of the Drawings (if filed)
  - Detailed Description
  - Claim(s)
  - Abstract of the Disclosure
4. ☒ Drawing(s) (35 U.S.C. 113) (Total Sheets )
5. ☒ Oath or Declaration (Total Pages )
  - a. ☐ Newly executed (original or copy)
  - b. ☐ Copy from a prior application (37 CFR 1.63 (d))  
(for a continuation/divisional with Box 18 completed)
  - i. ☐ **DELETION OF INVENTOR(S)**  
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☐ Application Data Sheet. See 37 CFR 1.76

## ADDRESS TO:

Assistant Commissioner for Patents  
Box Patent Application  
Washington, DC 20231

7. ☐ CD-ROM or CD-R in duplicate, large table or Computer Program (Appendix)
8. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
  - a. ☐ Computer Readable Form (CRF)
  - b. Specification Sequence Listing on:
    - i. ☐ CD-ROM or CD-R (2 copies); or
    - ii. ☐ paper
  - c. ☐ Statements verifying identity of above copies

## ACCOMPANYING APPLICATIONS PARTS

9. ☐ Assignment Papers (cover sheet & document(s))
10. ☐ 37 C.F.R. §3.73(b) Statement ☐ Power of Attorney  
(when there is an assignee)
11. ☐ English Translation Document (if applicable)
12. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
13. ☐ Preliminary Amendment
14. ☐ Return Receipt Postcard (MPEP 503)  
(Should be specifically itemized)
15. ☐ Certified Copy of Priority Document(s)  
(if foreign priority is claimed)
16. ☐ Non publication Request under 35 U.S.C. 122 (b)(2)(B)(i). Applicant must attach form PTO/SB/35 or its equivalent.
17. ☒ Other: Express Mail Certification

18. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment, or in an Application Data Sheet under 37 CFR 1.76:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: \_\_\_\_\_ / \_\_\_\_\_  
Prior application information: Examiner \_\_\_\_\_ Group / Art Unit: \_\_\_\_\_

For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

## 17. CORRESPONDENCE ADDRESS

☒ Customer Number or Bar Code Label

37490

or ☐ Correspondence address below

Name

John W. Carpenter

Address

Carpenter & Kulas, LLP  
1900 Embarcadero Road, Suite 109

City

Palo Alto

State

CA

Zip Code

94303

Country

USA

Telephone

650-842-0303

Fax

650-842-0304

Name (Print/Type)

John W. Carpenter

Registration No. (Attorney/Agent)

26,447

Signature



Date

August 6, 2003

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

15757 U.S. PTO  
10/635396  
08/06/03

16310 U.S. PTO  
08/06/03

PTO/SB/17 (11-00)

Approved for use through 10/31/2002. OMB 0651-0032

U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

# FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision.

## Complete if Known

Application Number: Unknown  
Filing Date: August 6, 2003  
First Named Inventor: John H. Crowe, et al.  
Examiner Name: Unknown  
Group / Art Unit: Unknown  
Attorney Docket No.: 010023-000510US

TOTAL AMOUNT OF PAYMENT (\$ 868.00)

## METHOD OF PAYMENT (check one)

1. ☐ The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:

Deposit  
Account  
Number

Deposit  
Account  
Name

☐ Charge Any Additional Fee Required  
Under 37 CFR 1.16 and 1.17  
☒ Applicant claims small entity status.  
See 37 CFR 1.27

2. ☒ Payment Enclosed:

☒ Check ☐ Credit card ☐ Money  
Order ☐ Other

## FEE CALCULATION

### 1. BASIC FILING FEE

| Large<br>Fee<br>Code | Entity<br>Fee<br>(\$) | Small<br>Fee<br>Code | Entity<br>Fee<br>(\$) | Fee Description        | Fee Paid |
|----------------------|-----------------------|----------------------|-----------------------|------------------------|----------|
| 101                  | 740                   | 201                  | 370                   | Utility filing fee     | 370.00   |
| 106                  | 330                   | 206                  | 165                   | Design filing fee      |          |
| 107                  | 510                   | 207                  | 255                   | Plant filing fee       |          |
| 108                  | 740                   | 208                  | 370                   | Reissue filing fee     |          |
| 114                  | 160                   | 214                  | 80                    | Provisional filing fee |          |

SUBTOTAL (1)

(\$ 370.00)

### 2. EXTRA CLAIM FEES

| Total Claims          | Extra<br>Claims | Fee from<br>below | Fee<br>Paid |
|-----------------------|-----------------|-------------------|-------------|
| 66                    | -20 = 46        | X 9               | = 414       |
| Independent<br>Claims | 5               | -3 = 2            | X 42 = 84   |
| Multiple<br>Dependent |                 | X                 | = 0         |

| Large<br>Fee<br>Code | Entity<br>Fee<br>(\$) | Small<br>Fee<br>Code | Entity<br>Fee<br>(\$) | Fee Description  |
|----------------------|-----------------------|----------------------|-----------------------|--|
| 103                  | 18                    | 203                  | 9                     | Claims in excess of 20                                     |
| 102                  | 84                    | 202                  | 42                    | Independent claims in excess of 3                          |
| 104                  | 280                   | 204                  | 140                   | Multiple dependent claim, if not paid                      |
| 109                  | 84                    | 209                  | 42                    | ** Reissue independent claims over original patent         |
| 110                  | 18                    | 210                  | 9                     | ** Reissue claims in excess of 20 and over original patent |

SUBTOTAL (2)

(\$ 498.00)

\*\*or number previously paid, if greater; For Reissues, see above

## FEE CALCULATION (continued)

| Large<br>Fee<br>Code | Entity<br>Fee<br>(\$) | Small<br>Fee<br>Code | Entity<br>Fee<br>(\$) | Fee Description  | Fee Paid |
|----------------------|-----------------------|----------------------|-----------------------|--|----------|
| 105                  | 130                   | 205                  | 65                    | Surcharge - late filing fee or oath  |          |
| 127                  | 50                    | 227                  | 25                    | Surcharge - late provisional filing fee or cover sheet                     |          |
| 139                  | 130                   | 139                  | 130                   | Non-English specification  |          |
| 147                  | 2,520                 | 147                  | 2,520                 | For filing a request for reexamination                                     |          |
| 112                  | 920*                  | 112                  | 920*                  | Requesting publication of SIR prior to Examiner action                     |          |
| 113                  | 1,840*                | 113                  | 1,840*                | Requesting publication of SIR after Examiner action                        |          |
| 115                  | 110                   | 215                  | 55                    | Extension for reply within first month                                     |          |
| 116                  | 400                   | 216                  | 200                   | Extension for reply within second month                                    |          |
| 117                  | 920                   | 217                  | 460                   | Extension for reply within third month                                     |          |
| 118                  | 1,440                 | 218                  | 720                   | Extension for reply within fourth month                                    |          |
| 128                  | 1,960                 | 228                  | 980                   | Extension for reply within fifth month                                     |          |
| 119                  | 320                   | 219                  | 160                   | Notice of Appeal   |          |
| 120                  | 320                   | 220                  | 160                   | Filing a brief in support of an appeal                                     |          |
| 121                  | 280                   | 221                  | 140                   | Request for oral hearing   |          |
| 138                  | 1,510                 | 138                  | 1,510                 | Petition to institute a public use proceeding                              |          |
| 140                  | 110                   | 240                  | 55                    | Petition to revive - unavoidable   |          |
| 141                  | 1,280                 | 241                  | 640                   | Petition to revive - unintentional   |          |
| 142                  | 1,280                 | 242                  | 640                   | Utility issue fee (or reissue)   |          |
| 143                  | 460                   | 243                  | 230                   | Design issue fee   |          |
| 144                  | 620                   | 244                  | 310                   | Plant issue fee  |          |
| 122                  | 130                   | 122                  | 130                   | Petitions to the Commissioner  |          |
| 123                  | 50                    | 123                  | 50                    | Petitions related to provisional applications                              |          |
| 126                  | 180                   | 126                  | 180                   | Submission of Information Disclosure Stmt                                  |          |
| 581                  | 40                    | 581                  | 40                    | Recording each patent assignment per property (times number of properties) |          |
| 146                  | 740                   | 246                  | 370                   | Filing a submission after final rejection (37 CFR § 1.129(a))              |          |
| 149                  | 740                   | 249                  | 370                   | For each additional invention to be examined (37 CFR § 1.129(b))           |          |
| 179                  | 740                   | 279                  | 370                   | Request for Continued Examination (RCE)                                    |          |
| 169                  | 900                   | 169                  | 900                   | Request for expedited examination of a design application                  |          |

Other fee (specify) \_\_\_\_\_

\*Reduced by Basic Filing Fee Paid

SUBTOTAL (3)

(\$)

## SUBMITTED BY

Name (Print/Type): John W. Carpenter  
Signature: *[Signature]*

Registration No.  
Attorney/Agent

26,447

Telephone

650-842-0303

Date

August 6, 2003

## Complete (if applicable)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

|  |  |
|--|--|
| <b>In Re Application of:</b><br><br>John H. Crowe, et al.<br><br><b>Application No.:</b> Unknown<br><br><b>Filing Date:</b> August 6, 2003<br><br><b>Title:</b> ERYTHROCYTIC CELLS AND<br>METHOD FOR LOADING SOLUTES | <b>Docket No.:</b> 010023-000510US<br><br><b>Re:</b> Filing Patent Application |
|--|--|

Commissioner for Patents  
 P.O. Box 1450  
 Alexandria, VA 22313-1450

**EXPRESS MAIL CERTIFICATE**

Utility Transmittal Letter;  
 Fee Transmittal Sheet;  
 Unsigned Declaration  
 Specification, Claims, Abstract and Drawings

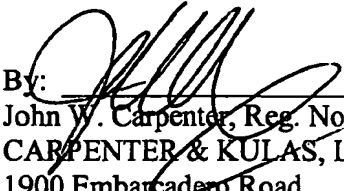
I hereby certify that this paper and the enclosures listed above are being deposited with the U.S. Postal Service "Express Mail to Addressee" Express Mail No.: EU121998647US on August 6, 2003, and is addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: August 6, 2003

Respectfully submitted,

EU 121998647 US

The Regents of the University of California

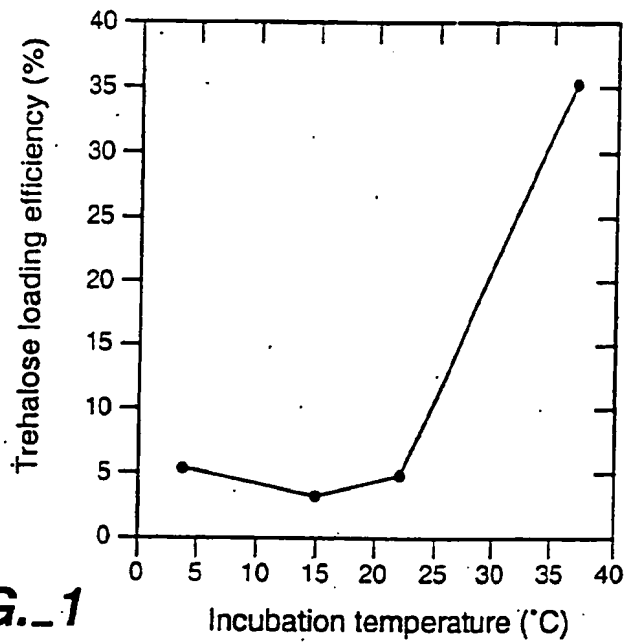
By:   
 John W. Carpenter, Reg. No. 26,447  
 CARPENTER & KULAS, LLP  
 1900 Embarcadero Road  
 Suite 109  
 Palo Alto, CA 94303  
 USA

**THIS PAGE BLANK (USPTO)**

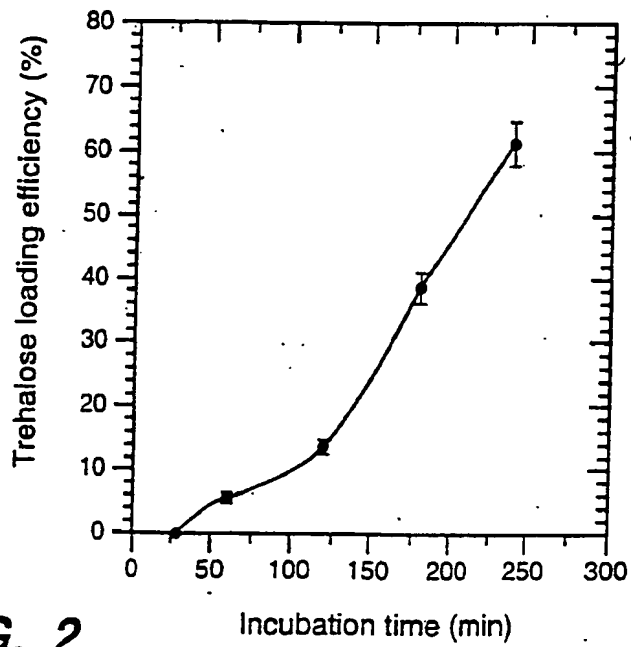
*ERYTHROCYTIC CELLS AND METHOD FOR  
LOADING SOLUTES*

Inventors: John H. Crowe et al.

Atty Docket No. 010023-000510US



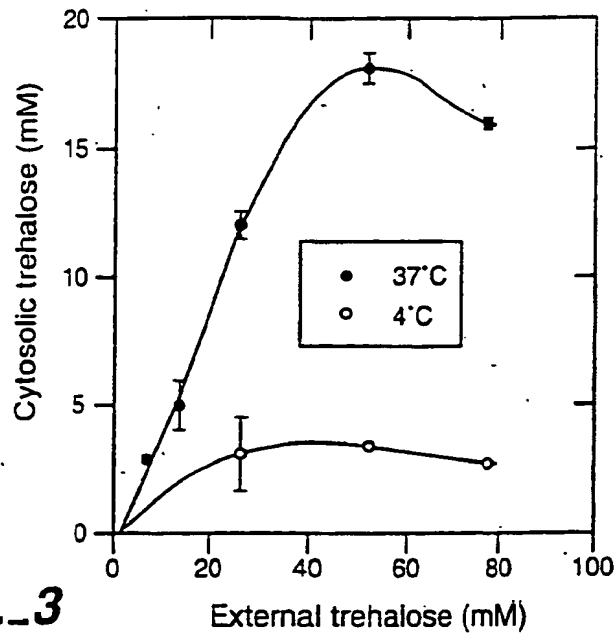
**FIG.\_1**



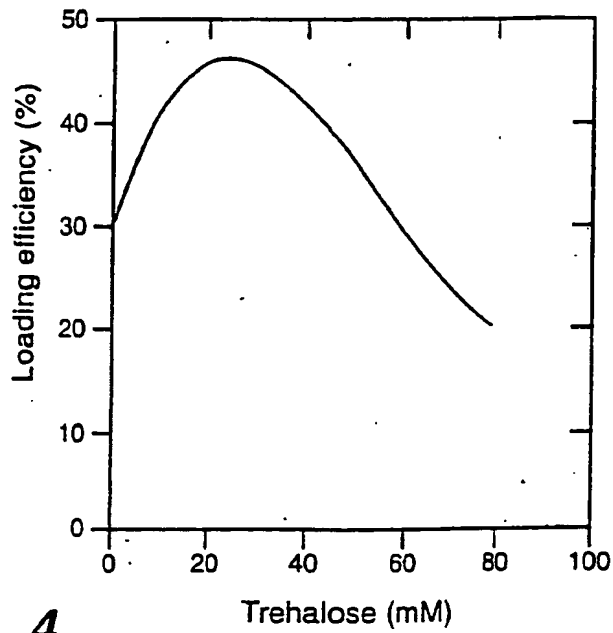
**FIG.\_2**

*ERYTHROCYTIC CELLS AND METHOD FOR  
LOADING SOLUTES*

Inventors: John H. Crowe et al.  
Atty Docket No. 010023-000510US



**FIG.\_3**



**FIG.\_4**



ERYTHROCYTIC CELLS AND METHOD FOR  
LOADING SOLUTES

Inventors: John H. Crowe et al.  
Atty Docket No. 010023-000510US

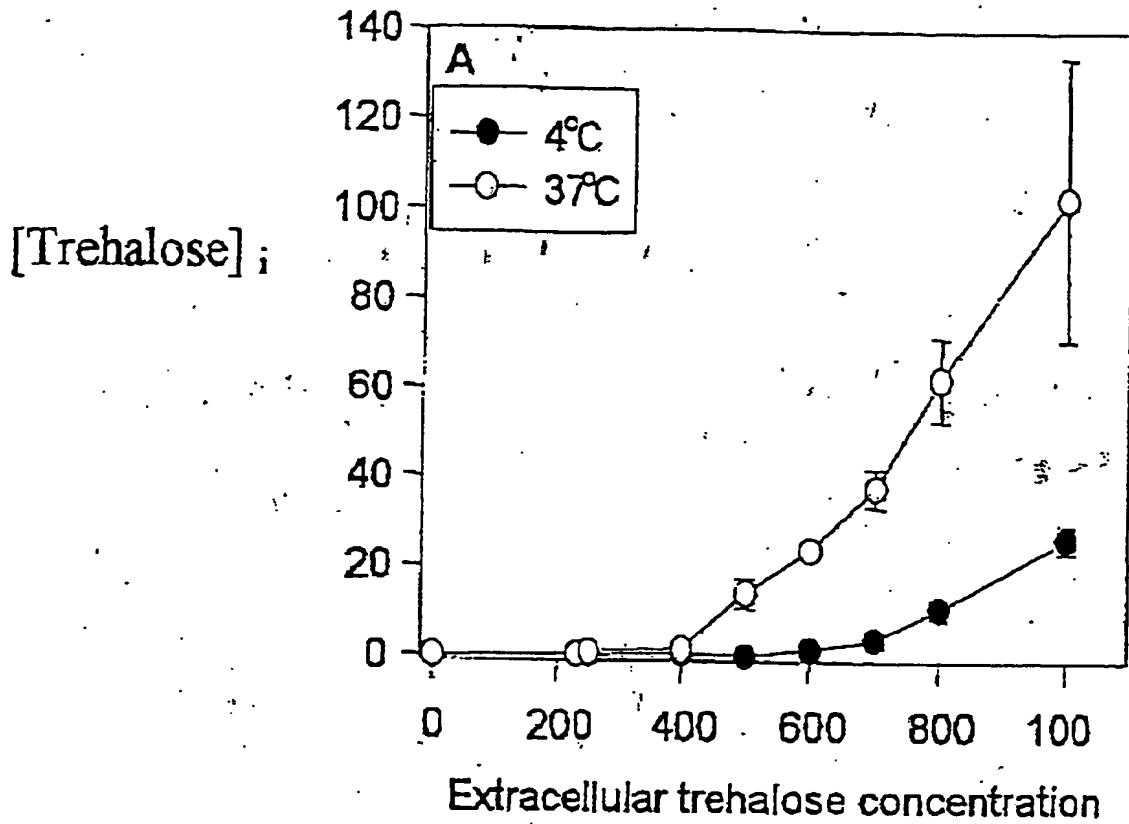


Fig. 5

ERYTHROCYTIC CELLS AND METHOD FOR  
LOADING SOLUTES

Inventors: John H. Crowe et al.  
Atty Docket No. 010023-000510US

**Fragility index of RBCs incubated overnight at 4 or 37°C  
in the presence of increasing trehalose concentrations**

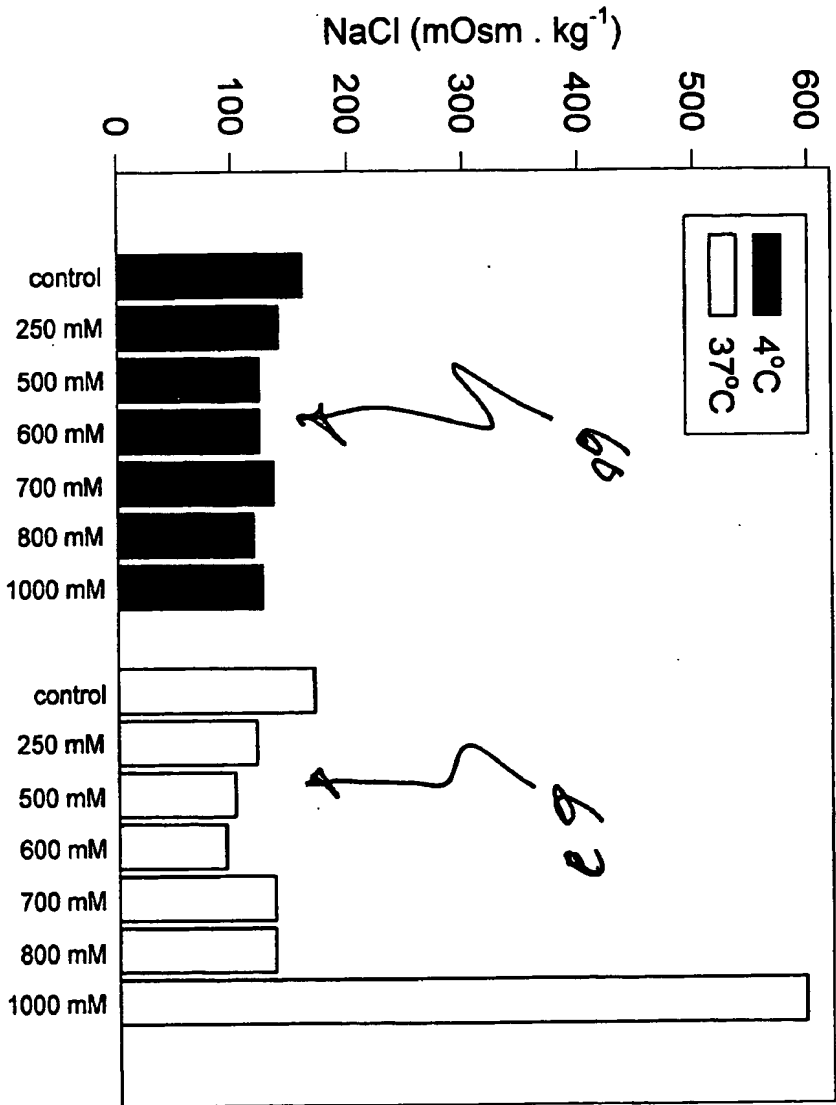


Figure 6

ERYTHROCYTIC CELLS AND METHOD FOR  
LOADING SOLUTES

Inventors: John H. Crowe et al.  
Atty Docket No. 010023-000510US

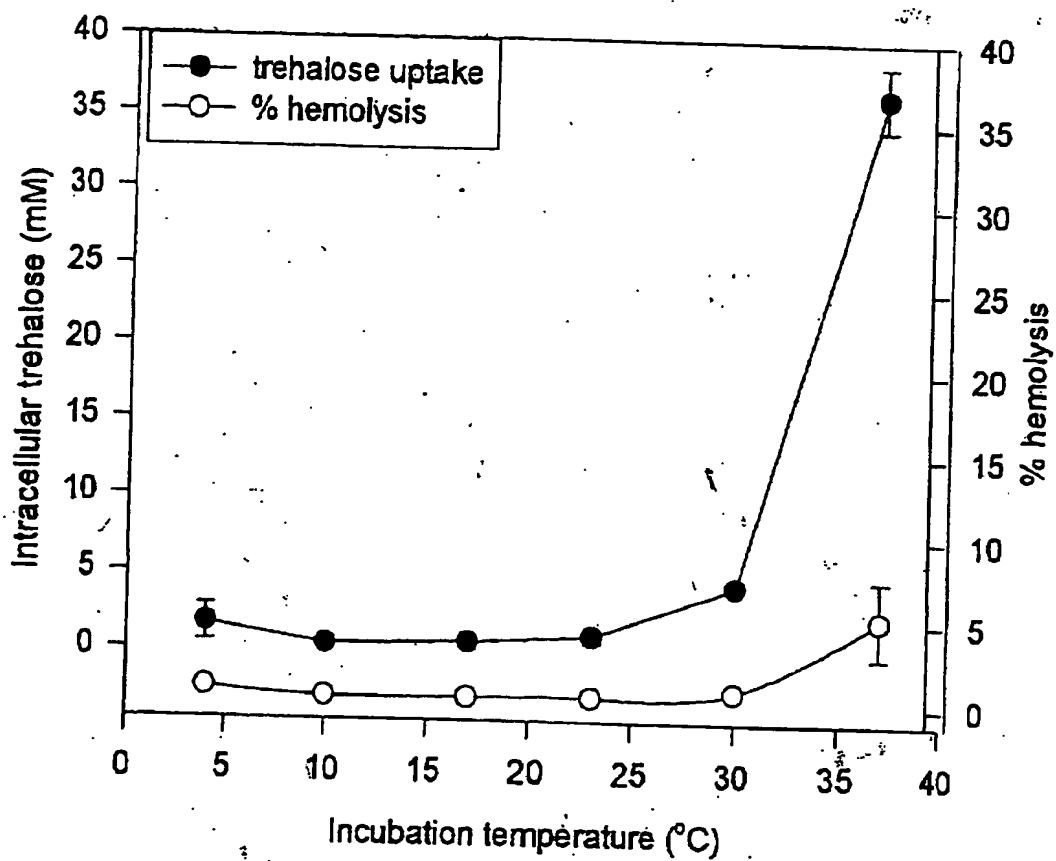
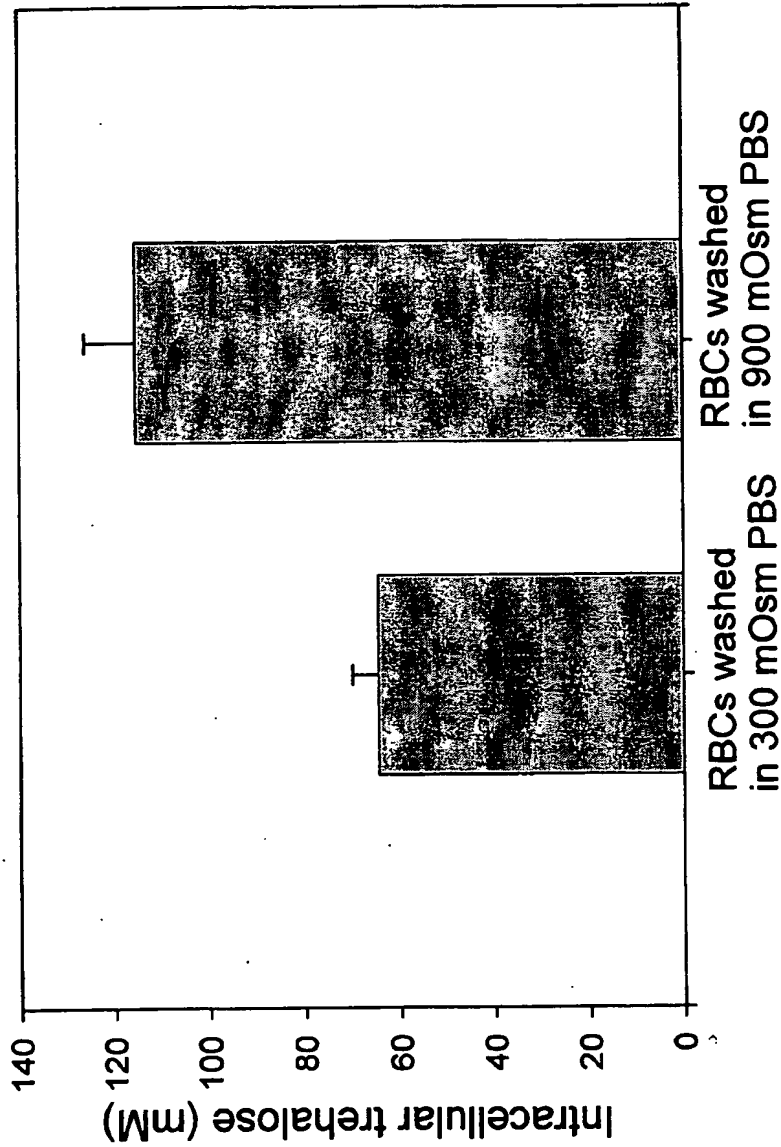


Fig 7

*ERYTHROCYTIC CELLS AND METHOD FOR  
LOADING SOLUTES*

Inventors: John H. Crowe et al.  
Atty Docket No. 010023-000510US

**Intracellular trehalose concentration as a function of the  
osmolarity of the washing buffer.**



**Figure 8**

# Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/025395

International filing date: 06 August 2004 (06.08.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US  
Number: 10/635,396  
Filing date: 06 August 2003 (06.08.2003)

Date of receipt at the International Bureau: 13 December 2004 (13.12.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland  
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☒ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☒ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**